ATTACHMENT A

E - X - A - M - P - L - E

O F

STANDARD OPERATING PROCEDURES (SOP)

SKELETAL EXAMINATIONS

1. SCOPE OF APPLICATION (LIMITATIONS)

This SOP describes the procedures and criteria for examining skeletal specimens.

2. PREREQUISITES

2.1 EQUIPMENT AND SUPPLIES

2.1.1 Equipment:
dissecting microscope with base illumination
shallow transparent dish
forceps
specimen containers (jars, trays, vials)

2.1.2 Chemicals:
examination solution [glycerin (1 part), 65% EtOH 1 part)]
glycerin for final storage

2.1.3 Supplies:
skeletal specimens (OP - Skeletal Processing)
data entry program (or Skeletal Examination data sheets)

3. CAUTIONARY NOTES OR SPECIAL CONSIDERATIONS

3.1 Wear protective gloves when directly handling specimens or solutions.
3.2 Use forceps to avoid direct contact with (and for easier manipulation of) specimens.

4. PROCEDURE

4.1 One person examines all specimens in a study. Examine all specimens without

knowledge of the specimen's treatment group. Identify each litter's treatment group only after all specimens in that study have been examined.

4.2 Use a dissecting microscope with a below-stage light source. In order to reduce glare, the specimens may be submerged in the solution the specimen is currently stored in (see OP - Skeletal processing). Use forceps to facilitate manipulation of the specimens.

4.3 Record findings using an appropriate computer program (e.g., RS/1); initial printouts of the data must be saved as raw data. Alternatively, the data may be recorded onto Skeletal Examination data sheets for later transcription into a computer file. **Note, in particular, the items listed below; however, do not limit the recording of findings to these items.

4.4 Examine the head:

4.4.1 Examine the mandible and orbits for size and position.

4.4.2 Note the ossification of the calvaria. Reduced ossification is indicated by a discrete boundary between degrees of ossification.

4.4.3 Examine the anterior region of the suture between the frontal bones for the (normal) absence of an "interfrontal bone" and score accordingly:
1 = absent
2 = barely visible (with normal magnification)
3 = readily visible
4 = severely enlarged

4.4.4 Score the size of the anterior fontanel (and the separation between the parietals):
1 = minimal
2 = slight (most typical for GD-20 rat fetuses)
3 = moderate
4 = severe

4.4.5 Score the ossification of the supraoccipital:
1 = >90% ossified
2 = 50-90% ossified
3 = >0 and <50% ossified
4 = completely unossified

4.4.6 Score the ossification of the hyoid:
1 = ossified (normal)
2 = reduced ossification
3 = completely unossified

4.4.7 Score palate closure. If necessary, break the mandibles for easier viewing.
1 = palatal shelves (virtually) touching
2 = gap between palatal shelves
3 = cleft palate

4.5 Examine the sternebrae. Identify by number (1=manubrium, 6=xyphoid) which

sternebrae are affected in the following categories: unossified; small, reduced ossification, or unilaterally ossified; bilobed or bipartite; offset. In addition, indicate the presence of an extra sternebra and identify any sternebrae that are fused and whether or not the fusion is associated with offsetting. Also indicate if offsetting is due to deficient ossification.

4.5.1 Designate a sternebra that is misaligned by at least one-half the sternebra's length as "offset."

4.5.2 If fusion between two (or more) sternebrae is present, indicate the sternebrae involved. If the fusion is associated with misalignment (i.e., offsetting), indicate this (e.g., "FO:3&4" indicates that sternebrae 3 and 4 are fused due to some offsetting of one or both sternebrae). Otherwise, "fused" indicates that the fusion was not associated with offsetting sternebrae (e.g., "FU:3&4").

4.6 Examine the ribs noting any fusions, bifurcations, or irregularities in shape (e.g., wavy) or ossification.

4.6.1 Note the presence (and side) of any cervical ribs. Indicate any cervical rib that do not articulate with vertebra C7.

4.6.2 If wavy ribs are predominant on the affected side(s), it is not necessary to identify each affected rib. For other findings, e.g., fused, specify the ribs involved.

4.6.3 Score the presence/length of the 13th thoracic (T13) rib and any lumbar ribs (posterior to T13) on each side:
0 = rib T13 short or absent
1 = lumbar rib absent (normal)
2 = focal lumbar rib, without length
3 = lumbar rib with length,
but less than one-half the length rib T13
4 = lumbar rib ≥ ½ the length of rib T13

4.7 Examine the vertebrae noting the shape and ossification of the centra and arches.

4.7.1 Record the numbers of centra that are bilobed and bipartite.

4.7.2 Except for unossified, bilobed, and bipartite centra, describe any findings with respect to the specific vertebra (e.g., S1 or sacral-1) and the side (R, L, or B) affected. For the purposes of identification, designate a vertebra associated with a 14th thoracic/lumbar rib as LU1.

4.7.3 Count the cervical vertebrae (and check for cervical ribs, see above). Count the number of presacral vertebrae (normally 26) and examine the lumbosacral junction. For specimens with a "hybrid" lumbosacral vertebra (i.e., showing lumbar characteristics on one side, but sacral characteristics on the other side), indicate the lumbar side. For example, "R" or "+R" indicates that the 27th vertebra is lumbar on the right and sacral on the left; "-R" indicates that the 26th vertebra is lumbar on the left and sacral on the right.

4.7.4 Count and record the number of ossified cervical vertebral centra.

4.7.5 Count and record the number of unossified thoracolumbosacral vertebral centra.

4.7.6 Count and record the number of ossified caudal vertebrae. Begin the count after the fourth sacral vertebra. For fetuses with a hybrid vertebra (see above), count the hybrid as the first sacral vertebra.

4.8 Examine the girdles and long bones. Note instances of unossified pubis, but not reduced ossification of the pubis.

4.9 Examine the feet for any fused or misshapen structures. For each foot, record the numbers of ossified metacarpals/metatarsals and proximal phalanges.

4.10 DATA PROCESSING: If data was handwritten on data sheets, transcribe the data for each fetus of each litter into a computer datafile; it may be entered into a program used for collection of raw data. Analyze the data using appropriate statistical methods (e.g., SAS or equivalent). Construct a table showing litter means and/or litter incidences of major findings, related findings, and observations that may be treatment related.

ATTACHMENT B

E - X - A - M - P - L - E

O F

STANDARD OPERATING PROCEDURES (SOP)

FETAL VISCERAL EXAMINATIONS

1. SCOPE OF APPLICATION (LIMITATIONS)

This SOP describes the procedures and criteria for examining visceral specimens.

2. PREREQUISITES

2.1 EQUIPMENT AND SUPPLIES

2.1.1 Equipment:

specimen jars or trays
jars or beakers
fume hood
dissecting microscope
forceps and iris scissors
cutting board or other cutting surface

2.1.2 Chemicals:

Bodian's solution
tap water

2.1.3 Supplies:

scalpels or razor blades
scintillation vials

CAUTIONARY NOTES OR SPECIAL CONSIDERATIONS

3.1 Wear protective gloves when directly handling specimens or fixatives.

3.2 Place specimens in tap water prior to sectioning; when the specimens sink, exposure to fumes from the fixative will be reduced. Specimens may remain in water for several days to further reduce exposure to fumes.

4. PROCEDURE

4.1 One person examines all specimens in a study. Examine all specimens without knowledge of the specimen's treatment group. Identify each litter's treatment group only after all specimens in that study have been examined. Note particularly the items described below; however, do not limit the recording of findings to these items. For Segment II studies, record the observations in the appropriate spaces on the Visceral Examination data sheet or enter the data in the appropriate computerized data entry program.

4.2 Maintaining litter identification, transfer specimens from Bodian's solution to an open container of tap water under a hood. When sunk, most of the fixative's fumes have dissipated; the specimen may then be removed from the hood for examination.

4.3 Examine each specimen externally for alterations of the head (shape, pinnae, eyes, eyelids, jaw, nares, lip), skin, tail, back, ventral wall, limbs, feet, and digits.

4.4 Section the specimen through the mouth (just below the pinnae) and the abdomen (leaving the diaphragm intact) yielding three portions: the head, forequarters and

hindquarters.

4.5 The head:

4.5.1 Remove the tongue and examine the palate for a cleft. Also examine the rugae for irregularities.

4.5.2 Make transverse sections perpendicular to the mouth and through the nasal passages, eyes, and the greatest diameter of the skull. In addition, make a mid-sagittal section through the hindbrain portion of the specimen. (The mid-sagittal section need not go entirely through the specimen; the two halves may remain "hinged" with connective tissue.)

***4.5.3** Examine the head sections with particular attention to the nasal passages and septum, palate and rugae, eyes, lenses, retinae, and ventricles of the brain.

***4.5.4** Score the dilation of the lateral ventricles:
1 = essentially none
2 = slight
3 = moderate
4 = severe

4.6 The forequarters:

4.6.1 Examine the liver for color and texture. Carefully remove it leaving the diaphragm intact.

4.6.2 Examine the diaphragm for herniation.

4.6.3 Cut through the length of the sternum and expose the thoracic cavity.

***4.6.4** Remove the thymus with forceps and examine the major blood vessels--in particular, note the presence of the innominate artery.

***4.6.5** Examine the trachea and esophagus by moving a forceps tip between the two from the larynx to the heart. In particular, note any fistulas and the right-left orientation.

4.6.6 Examine the lungs for size, lobation, and right-left orientation.

4.6.7 Examine the heart for size and orientation.

***4.6.8** Section the heart perpendicular to the ventricular septum or use ultramicrodissection scissors to cut along the pulmonary artery and right ventricle and then along the aorta and left ventricle.

***4.6.9** Examine the ventricular septum and valves.

***4.6.10** Examine the front feet.

4.7 The hindquarters:

4.7.1 Carefully remove the digestive viscera and spleen and note any signs of intra-abdominal hemorrhage.

***4.7.2** Examine the viscera for an accessory spleen or lobule at the caudate end of the spleen.

4.7.3 Examine the urinary bladder and the genitalia. Record the sex of

the fetus; note if the external genitalia do not correspond with the internal genitalia.

4.7.4 Examine the kidneys for size and position. If the anterior tip of the left kidney is below the level of the hilus of the right kidney, or if the posterior tip of the right kidney is above the level of the hilus of the left kidney, note the kidneys as offset. If the anterior tip of the left kidney is anterior to the anterior tip of the right kidney, note the kidneys as offset.

***4.7.5** Gently remove the mesentery covering the ureters. Then, score the widest dilation of each ureter:
1 = normal
2 = slight
3 = moderate
4 = severe

***4.7.6** Section each kidney transversely through the hilus and score the dilation of the renal pelvis/papilla size:
1 = papilla intact, no dilation
2 = papilla reduced
3 = papilla nearly absent
4 = papilla absent

***4.7.7** Examine the hind feet.

4.8 Place the sections of each specimen in a scintillation vial for storage; tap water should cover the tissues. Unless alterations were noted, the lower jaw and abdominal viscera (except for the urinary tract and genitalia) may be discarded. Label the vial/cap with the litter and fetus numbers of the specimen.

4.9 DATA PROCESSING: Enter the data for each fetus of each litter into a computer datafile and analyze the data using appropriate statistical methods, such as with SAS. Construct a table showing litter means and/or litter incidences of major findings, related findings, and observations that may be treatment related.

*Examine specimen with the aid of a microscope in this step.